

Crystal structure of cytochrome P-450cam complexed with the (1S)-camphor enantiomer

Ilme Schlichting^a, Christiane Jung^{b,*}, Heike Schulze^b

^aMax Planck Institute for Molecular Physiology, Division for Physical Biochemistry, Rheinlanddamm 201, 44139 Dortmund, Germany

^bMax Delbrück Center for Molecular Medicine, Robert-Rössle-Strasse 10, 13125 Berlin, Germany

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Abstract The crystal structure of cytochrome P-450cam complexed with the enantiomer (1S)-camphor has been solved to 1.8 Å resolution and compared with the structure of the (1R)-camphor P-450cam complex. The overall protein structure is the same for both enantiomer complexes. However, the orientation of the substrates in the heme pocket differs. In contrast to (1R)-camphor, the (1S)-enantiomer binds in at least two orientations. The major binding mode of (1S)-camphor resembles the one of the (1R)-enantiomer in that there is a hydrogen bond between Tyr-96 and the quinone group of camphor, and the 10-methyl group points towards the I-helix. The binding differs in that C-5 is not at a position suitable for hydroxylation. In the other orientation (1S)-camphor is not hydrogen bonded, but C-5 is located suitably for hydroxylation.

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Key words: Cytochrome P-450cam; Crystal structure; Substrate interaction

1. Introduction

Cytochrome P-450s are monooxygenases that form a family of ubiquitous heme proteins. P-450s are unique in their ability to hydroxylate non-activated carbon atoms. This makes them an attractive target for environmental or pharmaceutical industries. However, a prerequisite for tailoring specific functions for application in e.g. bioremediation is to understand the structure activity relationship between target molecule and enzyme. To probe the information that can be obtained from biophysical data we studied the interaction between P-450cam and its natural substrate (1R)-camphor, and its optical antipode (1S)-camphor, respectively. The two camphor isomers are an ideal test object for such a study since they have the same chemical characteristics such as polarizability, dipole moment, etc. Thus, differences in their biophysical characteristics are solely due to changes in binding mode of the substrate.

Cytochrome P-450cam (CYP 101 [1]) from *Pseudomonas putida* catalyzes the hydroxylation of camphor to 5-exo-hydroxy-camphor. Stereo and regio specificity is obtained by a snug fit of (1R)-camphor into the heme pocket. This is provided by numerous van der Waals interactions between the camphor methyl groups and the protein as well as a hydrogen

bond between the quinone group of camphor and the hydroxyl group of Tyr-96. In the case of (1S)-camphor, however, the kinetics and thermodynamics of the heme iron spin state transition of oxidized P-450 camphor complexes indicate a higher solvent accessibility and substrate mobility [2]. This finding is corroborated by time-resolved infrared spectroscopy which shows faster rebinding kinetics of the photodissociated CO to the P-450cam (1S)-camphor complex [3]. We interpreted the data such that a modified binding geometry of (1S)-camphor in the active site, e.g. a missing contact of the 10-methyl group of (1S)-camphor to the I-helix, is the major cause for the higher accessibility of the protein structure for water molecules and hence for the increased substrate mobility [4]. It has been argued that this higher substrate mobility might be the reason for the increased amount of uncoupling for the (1S)-camphor bound P-450cam (11%) compared to the (1R)-camphor complex (3–4%) [5]. To test these interpretations we solved the crystal structure of P-450cam bound with (1S)-camphor. The structural data confirm most of our previous predictions on (1S)-camphor binding.

2. Materials and methods

Cytochrome P-450cam from *Pseudomonas putida* expressed in *Escherichia coli* TB1 was isolated and purified to an absorbance ratio 392 nm/280 nm of 1.2 according to Jung et al. [6]. To obtain the substrate-free protein the natural substrate (1R)-camphor was removed by dialysis and column chromatography as previously described [6]. The concentrated substrate-free protein was dialyzed over night with several buffer exchanges against 50 mM Tris-HCl buffer, pH 7.4, 250 mM KCl and 1 mM (1S)-camphor. Then the protein was concentrated by ultrafiltration to approximately 1 mM. To 220 µl of this concentrated (1S)-camphor bound P-450cam solution 2.3 µl of a 700 mM ethanolic (1S)-camphor stock solution were added. The completeness of substrate binding was verified by the optical spectrum of the Soret band which indicated an almost complete shift of the iron spin state to the high spin state at room temperature. (1S)-camphor was purchased from Merck and Sigma and used without further purification.

The P-450cam (1S)-camphor complex was co-crystallized in the presence of excess (1S)-camphor at 4°C by dialyzing 25 µl of a 1 mM P-450cam(1S) solution against 50 mM Tris-HCl pH 7.4, 250 mM KCl, 100 mM DTE, 10% PEG 8000. This procedure (a modification of the one described by Poulos et al. [7]) produces two crystal forms: large tetragonal bipyramids, and long orthorhombic needles that have hollow pyramidal cavities. The latter form was used for the structure determination of the P-450cam(1S) complex. Diffraction data were collected at beamline X12C at the Brookhaven synchrotron using a MAR detector and a wavelength of 0.95 Å. The data were reduced with DENZO and SCALEPACK [8]; the data statistics are summarized in Table 1. The coordinates of the P-450cam(1R) complex in the tetragonal crystal form (unpublished) were used as a starting model for the molecular replacement calculations using AMORE [9]. Refinement (including bulk solvent correction and individual temperature factor refinement) was carried out with X-PLOR 3.851 [10]. After several rounds of interactive model fitting using O [11], placement of water molecules and refinement, the density of (1S)-camphor

*Corresponding author. Fax: +49 (30) 94063329. Correspondence may also be sent to I.S. [Fax +49 (231) 1206622].

Abbreviations: P-450cam, cytochrome P-450cam from *Pseudomonas putida* (CYP101); P-450cam(1R), (1R)-camphor bound form of cytochrome P-450cam; P-450cam(1S), (1S)-camphor bound form of cytochrome P-450cam; DTE, dithioerythriol; PEG, polyethylene glycol

was modeled. It was obvious from the electron density that there was more than one orientation present. Two orientations of (1S)-camphor were fitted into the density (see Fig. 1), and their occupancies and temperature factors were refined as groups. They converged to 50%, 25.1 Å² and 20%, 40.5 Å², respectively. We cannot rule out the presence of a partially occupied water molecule bound to the heme iron. When fixing the occupancies to 70% and 30% the group temperature factors converged to 40.9 Å² and 100 Å², respectively. The structure was refined to 1.8 Å resolution to a final R-factor of 20.9% and R_{free} of 26.5%. The refinement statistics are shown in Table 1.

3. Results

The crystal structure of the P-450cam(1S) complex was solved from a new orthorhombic crystal form grown from polyethylene glycol. The molecular replacement method was used for structure determination using the structure of P-450cam complexed to (1R)-camphor as a search model. Table 1 gives the summary of the statistics of the diffraction data and of the crystallographic refinement. Apart from surface loops there are no remarkable changes in the protein structure in the P-450cam (1R)- or (1S)-camphor complexes. However, the substrate orientation in the heme pocket is very different for both enantiomers. While (1R)-camphor shows one distinct orientation with a hydrogen bond between the substrate quinone group and the Tyr-96 hydroxyl, (1S)-camphor binds in at least two orientations. The major conformation with a population of approximately 50% is hydrogen bonded to Tyr-96 and has most of its methyl groups within 0.6 Å as (1R)-camphor. However, the C-5 carbon atom where the hydroxylation takes place is not oriented towards the heme iron as in the (1R)-camphor geometry. The minor orientation of (1S)-camphor, whose occupancy refines to 20%, is not hydrogen bonded to the hydroxyl group of Tyr-96, the C-5 carbon atom points towards the heme iron, and most of the methyl

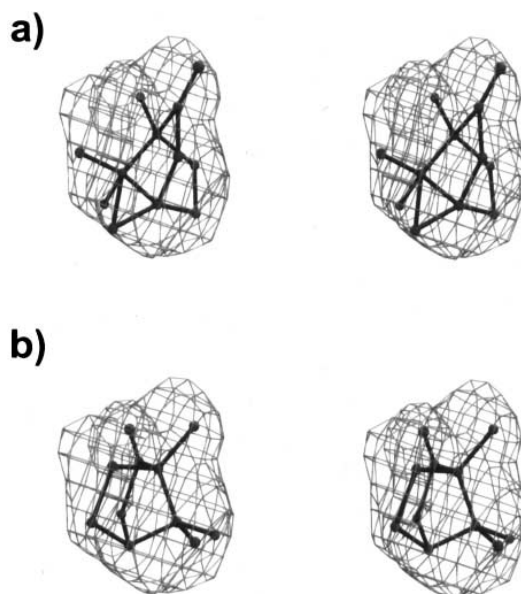


Fig. 1. Stereoview of the two orientations of (1S)-camphor in a $(3F_{\text{obs}} - 2F_{\text{calc}})$ electron density map. The electron density is shown at a σ -level of 1.0. The higher occupied orientation (corresponding to 'first orientation' in Tables 2 and 3) is shown in a. The lower occupied orientation (corresponding to 'second orientation' in Tables 2 and 3) is shown in b. The orientation of the camphor molecules is the same as in Fig. 2. The figure was generated using MOLSCRIPT [14].

groups have different locations as in (1R)-camphor. The unfavorable interactions between the quinone oxygen and the methyl group of Val-247, and the C-10-methyl group and the hydroxyl of Tyr-96 are reflected in the high temperature factors of this (1S)-camphor orientation. The spatial correspondences (i.e. which atoms of (1S)-camphor are located at a similar position to atoms of (1R)-camphor) of the camphor atoms of P-450 bound (1R)-camphor and the two (1S)-camphor binding sites are listed in Table 2. Table 3 gives the distances between the substrate atoms, relevant amino acids of the protein and the heme. Fig. 1 shows the fit of the two orientations of (1S)-camphor in the electron density map, Fig. 2a–c show the binding modes of (1R)-camphor and (1S)-camphor in the heme pocket.

4. Discussion

The stereoisomers of camphor provide a subtle and specific tool to probe the active site in cytochrome P-450cam. Since both enantiomers have the same physico-chemical properties such as mass, dipole moment, polarizability and solubility in non-chiral solvents, it is only the different sterical arrangement of the two enantiomers in the heme pocket that produces the differences in the spectral and enzymatic activity parameters that we and others reported recently. To summarize briefly, we observed that (i) the spin reaction volume difference measured using the high pressure technique is smaller by $16 \pm 9 \text{ cm}^3/\text{mol}$ for P-450cam(1S) compared to P-450cam(1R); (ii) the amount of high spin state population trapped in temperature jump (298 K to 77 K) experiments is smaller for the (1S)-camphor complex; (iii) the half transition temperature of the thermal unfolding of 53.8°C for the (1S)-camphor bound oxidized cytochrome P-450cam is one degree lower than the

Table 1
Data collection and refinement statistics

Diffraction data	
Resolution (Å)	1.8
Observed reflections	143928
Unique reflections	41095
Completeness (% , overall/last shell (1.86–1.8 Å))	92.0/69.4
R _{sym} ^a (% , overall/last shell)	4.6/28.7
Space group and unit cell (Å)	P2 ₁ 2 ₁ 2 ₁ , a = 64.3, b = 66.2, c = 106.8
Molecules/asymmetric unit	1
Refinement	
Resolution (Å)	26–1.8
R _{factor} ^b /R _{free} (%)	20.9/26.5
RMS deviations	
bond lengths (Å)	0.009
bond angles (Å)	1.22
dihedral angles (°)	34.4
improper angles (°)	1.62
# water molecules	278
Average B(Å ²)	
main chain, side chain	26.5, 30.0
(1S)-camphor position 1 (occ. (%), B, (Å ²))	50, 25.1
(1S)-camphor position 2 (occ. (%), B, (Å ²))	20, 40.5

^aR_{sym} = $|I - \langle I \rangle| / I$.

^bR_{factor} = $\|F_{\text{obs}}\| - \|F_{\text{calc}}\| / \|F_{\text{obs}}\|$, (5% randomly omitted reflections were used for R_{free}).

Table 2

Spatial correspondence between atoms in (1R)-camphor and the two orientations found for (1S)-camphor

(1R)-camphor	(1S)-camphor	First orientation ^a	(1S)-camphor	Second orientation ^a
C1	C1	0.3	C1	0.6
C2	C2	0.5	C1, C10	1.1
C3	C3	0.4	C9	0.6
C4	C4	0.6	C4	1.2
C5	C8	0.7	C5	0.5
C6	C8	1.1	C6	0.7
C7	C7	1.1	C4, C7	0.8, 1.1
C8	C5	0.6	C8	0.9
C9	C9	0.6	C3	1.2
C10	C10	0.7	O	0.9
O	O	0.4	C10	0.8

^aThe distances (given in Å) refer to the corresponding atoms in (1R)-camphor.

value for the (1R)-camphor bound protein; (iv) in the CO bound form of P-450cam at 290 K the (1S)-camphor complex reveals another CO stretch vibration population distribution with slightly higher frequencies compared to the (1R)-camphor complex [2] and the rebinding kinetics of the photodissociated CO ligand is faster for the P-450cam(1S) [3]. Furthermore, (1S)-camphor binds to P-450cam with a higher dissociation constant [2]. These findings were explained by an incomplete fit between the methyl groups of the (1S)-substrate and the protein, in particular by a missing contact of the 10-methyl group to the I-helix, causing a higher substrate mobility and water accessibility to the protein [2,4]. A more specific model for (1S)-camphor binding was proposed on the ground that both enantiomers are solely hydroxylated at the 5-exo position, with (1S)-camphor showing increased uncou-

pling in the P-450cam catalyzed substrate conversion [5,12]. Thus, it was suggested that both (1R)-camphor and (1S)-camphor binding is conducted by the hydrogen bond between its quinone oxygen and the hydroxyl of Tyr-96 such that C-5 is positioned at the same location as in (1R)-camphor and that the protons at the C-3 position occupy the position where the 10-methyl group is located in (1R)-camphor. Molecular dynamics simulations of the (1S)-camphor binding mode were also based on the assumption that the hydrogen bond is maintained and show an increased mobility as reflected in a root mean square displacement of 0.9 Å in comparison to 0.58 Å for (1R)-camphor [2].

The crystal structure of the P-450cam (1S)-camphor complex presented here allows to test these predictions. Since a new crystal form was used the structure was solved by the

Table 3

Protein and heme atoms within 4 Å to (1R)-camphor and (1S)-camphor atoms, respectively

Protein and heme atoms	(1R)-camphor	(1S)-camphor, first orientation	(1S)-camphor, second orientation
87-Phe-CE2	O 3.7		C10 4.0
87-Phe-CZ	O 3.6	O 3.7	
96-Tyr-CE2	O 3.4	O 3.7	
96-Tyr-CZ	O 3.4	O 3.7	
96-Tyr-OH	C2 3.4	C2 3.7	
96-Tyr-OH	C3 3.6	C3 3.8	
96-Tyr-OH	O 2.6	O 2.9	C10 3.3
101-Thr-OG1			C9 4.0
101-Thr-CG2			C9 4.0
185-Thr-CG2	C10 3.9		O 3.7
244-Leu-CD1	C2 3.7	C2 4.0	
244-Leu-CD1	C3 3.8	C3 3.9	C6 3.8
244-Leu-CD1	O 3.7	O 3.8	C10 3.7
247-Val-CG1	C6 3.9		
247-Val-CG1	C10 3.7	C10 3.6	O 3.4
248-Gly-CA			C8 4.0
252-Thr-CG2			C9 3.7
295-Val-CG1	C9 3.9		
295-Val-CG1	C8 3.6	C5 3.8	
295-Val-CG2	C9 3.9		
297-Asp-OD2	C8 3.7		C8 3.7
396-Val-CG2			C3 3.9
396-Val-CG2			O 3.9
417-Hem-FE			C5 3.9
417-Hem-NA	C4 3.8		C4 4.0
417-Hem-NA	C5 4.0		C5 3.9
417-Hem-ND	C5 3.6		C5 3.9
417-Hem-C1A	C4 3.6	C4 3.8	
417-Hem-CBA		C5 4.0	C9 3.6
417-Hem-CHA	C4 3.8		C9 4.0
417-Hem-C4D	C4 4.0		
417-Hem-C4D	C5 3.9		
417-Hem-C4A	C9 3.9		

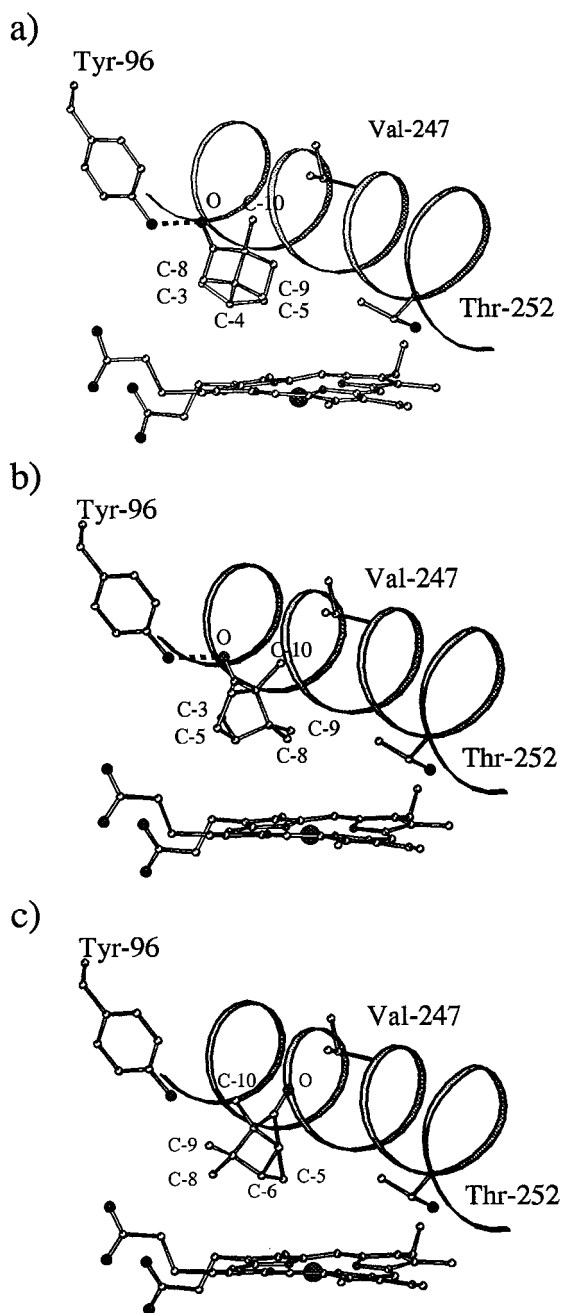


Fig. 2. View of the two (1S)-camphor conformations (b, c) in the heme pocket in comparison to the (1R)-camphor orientation (a). The higher occupied orientation of (1S)-camphor (corresponding to 'first orientation' in Tables 2 and 3) is shown in b. Camphor is shown in relation to the heme, part of the I-helix and Tyr-96. The figure was generated using MOLSCRIPT [14].

molecular replacement method. Apart from some surface loops the structure of the protein complexed to either camphor isomer is the same in the two crystal forms within our coordinate error. However, in contrast to (1R)-camphor, (1S)-camphor binds in at least two orientations. The higher occupied conformation is hydrogen bonded to Tyr-96 as suggested by Kadkhodayan et al. [5] and by us [2,4] but C-5 is oriented versus Val-295 ($\beta 3$ -sheet) and occupies the position taken by C-9 in (1R)-camphor. Thus, this conformation has not the right orientation for hydroxylation at C-5. The slightly different positions of the 9- and 10-methyl groups in this orienta-

tion compared to (1R)-camphor suggest a weaker contact to the I-helix, however, not as weak as we originally expected [2,4]. The second binding mode of (1S)-camphor should be catalytically competent as C-5 is at the same location as in (1R)-camphor. This is made possible by the absence of a hydrogen bond to Tyr-96. The loss of the Tyr-96 camphor interaction might result in less restriction of the mobility of Tyr-96. This may explain the increase in the cation dissociation constant in the P-450cam (1S)-camphor complex [2] since the carbonyl of Tyr-96 is part of the cation binding site.

Despite the differences in binding between (1R)-camphor and (1S)-camphor observed in the crystal structure, the product of the P-450 catalyzed hydroxylation, 5-exo-hydroxy-camphor, is formed with essentially the same yield (100% versus 97.8%, [5]) although at a reduced rate (2315 nmol/min/nmol P-450 versus 1246 nmol/min/nmol P-450, [5]). Since the lower occupied (1S)-camphor arrangement has the C-5 carbon atom in the correct orientation to the heme iron one may conclude that the main product (C-5 alcohol) results from the C-5 hydroxylation in this conformation. However, the population of 20% of this conformation cannot be assigned quantitatively to the 89% of the NADH consumption leading to the C-5 alcohol. In contrast, the total NADH consumption for the (1S)-camphor P-450 complex corresponds with 93% almost to that of the (1R)-camphor system [5]. Thus, the main activity difference of P-450cam complexed with (1S)-camphor compared to its enantiomer (1R)-camphor is the increased amount of uncoupling hydrogen peroxide formation (11% versus 4%) [5,12] which also cannot be assigned quantitatively to the 50% population of the less favorable (1S)-camphor orientation. In summary, the quantitative assignment of the (1S)-camphor orientations in the active site of oxidized P-450 remains still an open question.

For the reduced P-450 in its carbon monoxide complex, which might model the physiologically relevant dioxygen complex, we have recently shown that the (1S)-camphor complex has two subconformers with CO stretch vibration frequencies at 1940.2 cm^{-1} and 1946.3 cm^{-1} at room temperature, respectively [2]. These frequencies are higher than the values observed for the (1R)-camphor complex (1930 cm^{-1} and 1939.7 cm^{-1}). We found for a large number of substrates that an increased CO stretch vibration frequency is related to a higher water content which compensates polar interactions of the iron CO (O_2) ligand to the Thr-252 region in the I-helix [13]. The higher water accessibility is probably caused by the higher substrate mobility which is reflected in the occurrence of at least these two orientations of (1S)-camphor seen in the crystal. In addition, the interactions between the protein and the weaker occupied orientation of (1S)-camphor are not very favorable (e.g. quinone oxygen pointing towards the methyl groups of Val-247 and Thr-185, respectively, and C-10 pointing towards the hydroxyl of Tyr-96). A loosened contact to the I-helix might favor the leaving of oxygen as hydrogen peroxide over the splitting of the O-O bond and the formation of the hydroxylating Fe-O species. A higher mobility in the heme pocket connected with an increased water accessibility and an increased amount of uncoupling has also been reported and discussed for other camphor analogues [15].

The coordinates and structure factors of the P-450cam(1S) complex have been submitted to the Brookhaven data base. The access code is 1akd.

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References

- [1] Nelson, D.R., Kamataki, T., Waxman, D.J., Guengerich, F.P., Estabrook, R.W., Feyereisen, R., Gonzalez, F.J., Coon, M.J., Gunsalus, I.C. and Gotoh, O. (1993) *DNA Cell Biol.* 12, 1–51.
- [2] Schulze, H., Hui Bon Hoa, G., Helms, V., Wade, R.C. and Jung, C. (1996) *Biochemistry* 35, 14127–14138.
- [3] Contzen, J., Ristau, O. and Jung, C. (1996) *FEBS Lett.* 383, 13–17.
- [4] Schulze, H., Hui Bon Hoa, G. and Jung, C. (1997) *Biochim. Biophys. Acta* 1338, 77–92.
- [5] Kadkhodayan, S., Coulter, E.D., Maryniak, D.M., Bryson, T.A. and Dawson, J.H. (1995) *J. Biol. Chem.* 270, 28042–28048.
- [6] Jung, C., Hui Bon Hoa, G., Schröder, K.-L., Simon, M. and Doucet, J.P. (1992) *Biochemistry* 31, 12855–12862.
- [7] Poulos, T.L., Perez, M. and Wagner, G.C. (1982) *J. Biol. Chem.* 257, 10427–10429.
- [8] Otwinowski, Z. (1992) in: *An Oscillation Data Processing Suite for Macromolecular Crystallography*, Yale University Press, New Haven, CT.
- [9] CCP4: SERC Collaborative Computing Project No. 4 (Warrington, UK, 1979).
- [10] Brnger, A. (1992) *X-PLOR Version 3.1: A System for X-ray Crystallography and NMR*, Yale University Press, New Haven, CT.
- [11] Jones, T.A., Zhou, J.-Y., Cowan, S.W. and Kjeldgaard, M. (1991) *Acta Cryst. A* 47, 110–119.
- [12] Maryniak, D.M., Kadkhodayan, S., Crull, G.B., Bryson, T.A. and Dawson, J.H. (1993) *Tetrahedron* 49, 9373.
- [13] Jung, C., Schulze, H. and Deprez, E. (1996) *Biochemistry* 35, 15088–15094.
- [14] Kraulis, P.J. (1991) *J. Appl. Cryst.* 24, 946–950.
- [15] Raag, R. and Poulos, T.L. (1991) *Biochemistry* 30, 2674–2684.